Supporting Information

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SI Methods

Cells, Viruses, and Plasmids. Vero E6, BHK, and 293T cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (Cellgro) containing 10% FBS, 100 units/mL penicillin, and 10 μg/mL streptomycin.

Infectious bronchitis virus strains Beaudette and Massachusetts 41 (M41) were grown in specific pathogen-free, 10-day-old embryonated chicken eggs, ($\approx 10^2$ egg infectious $doses_{50}$ per egg), and the allantoic fluid was harvested at 24 h (IBV Beaudette) or 48 h (IBV M41) after inoculation. The allantoic fluid was clarified by centrifugation at 2,000 $\times$ *g* for 15 min and then centrifuged at 18,000 rpm in an SW32 rotor (Sorvall). The pellet was gently resuspended as a 2 mg/mL stock in PBS.

A plasmid expressing the codon-optimized version of the wild-type SARS-CoV S protein, containing a C-terminal C9 epitope tag (pcDNA3.1(-)/SARS-S), was kindly provided by Michael Farzan (1). Mutations and insertions were generated by PCR mutagenesis, and all of the constructs were verified by DNA sequencing. Mutants containing furin cleavage sites were expressed with the vector pEF4/myc-His (Invitrogen).

Western Blots and Cell Surface Biotinylation. BHK cells expressing the SARS wild type or SARS-R667N were lysed with RIPA buffer containing protease inhibitor mixture (Roche). Cell lysates were diluted with concentrated SDS/PAGE sample buffer containing DTT, incubated for about 5 min at 95 °C, and run on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose. Blocking and antibody incubations were carried out in TBS containing 3% BSA and 0.1% Nonidet P-40. Immunoreactive bands were identified by using horseradish peroxidase-conjugated secondary antibodies, and detection was carried out by using chemiluminescence (Pierce).

For cell surface biotinylation, BHK cells were transfected with plasmids encoding the different mutants and incubated for 24 h at 32 °C. Cells were washed on ice with cold PBS and incubated twice with 250 μg/mL EZ link sulfo-NHS-SS-biotin (Pierce) in PBS for 15 min each. Unreacted biotin was quenched with 2 washes with 50 mM glycine in PBS. Cells were lysed with RIPA buffer containing protease inhibitors, and biotinylated proteins were precipitated with streptavidin-agarose beads (Sigma). After washing, biotinylated proteins were recovered by addition of SDS/PAGE sample buffer. Cell surface SARS-CoV proteins were detected in Western blot by using anti-C9 or anti-myc antibodies. Images were obtained with an LAS-3000 Fujifilm imaging system (Fuji Photo Film Co. Ltd.), and bands were quantified by using Image Gauge software (Fuji Photo Film Co. Ltd.).

Statistical Analyses. Data were analyzed with GraphPad Prism software (version 3.0). *P* values are summarized as follows: ***, *P* value extremely significant ($P < 0.001$); **, *P* value very significant ($P = 0.001$ to 0.01); and *, *P* value significant ($P = 0.01$ to 0.05).

1. Bartosch B, Dubuisson J, Cosset FL (2003) Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes. *J Exp Med* 197:633–642.

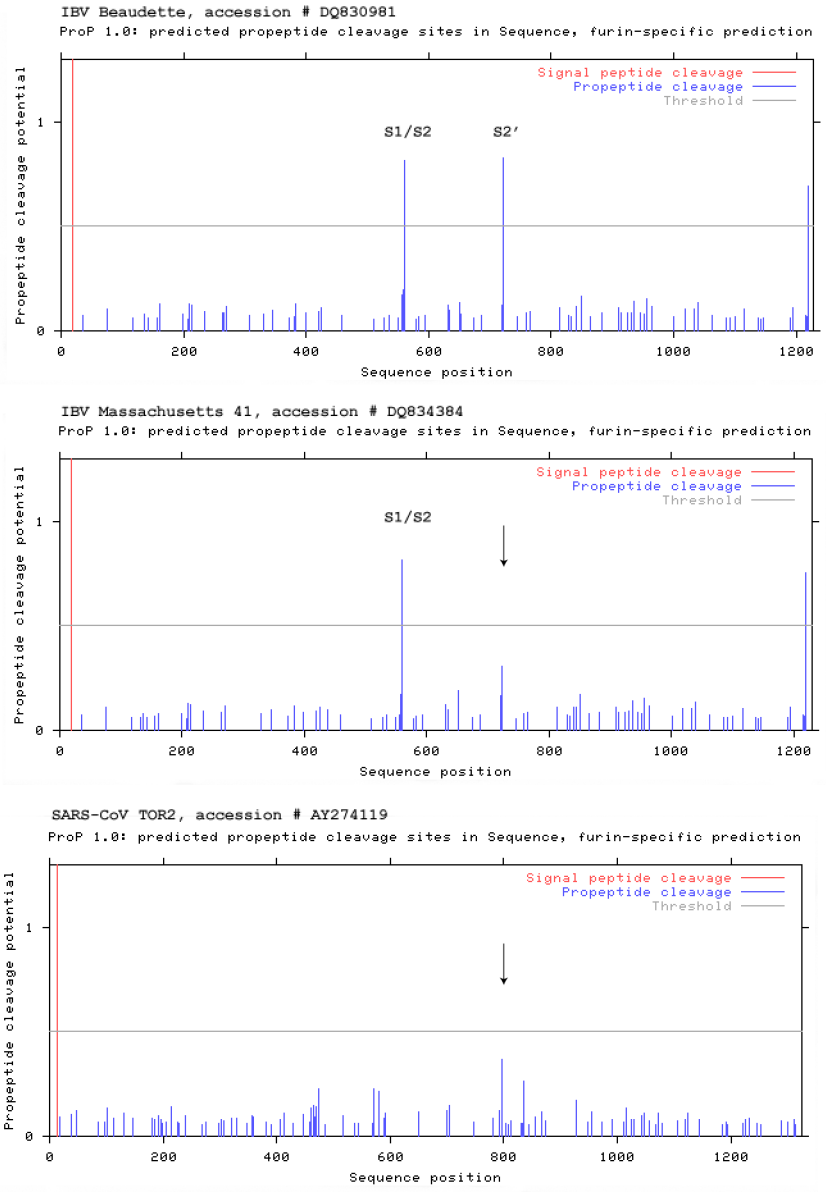

Fig. S1. Identification of additional cleavage sites within the coronavirus S protein. Prediction of cleavage sites for the spike proteins of IBV strain Bdtt, IBV strain M41, and SARS-CoV, strain Tor2 was carried out by using the ProP 1.0 server (www.cbs.dtu.dk/services/ProP/), using the furin-specific prediction as the default. Signal peptide cleavage sites are also shown, based on integration of the ProP server with the SignalP server (www.cbs.dtu.dk/services/SignalP-2.0/). The position of the S1–S2 boundary and the predicted S2′ cleavage site is indicated, with the location of S2′ shown with an arrow for M41 and Tor2.

A

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Beaudette IBV 5a                STGEFNISLLLTTPSSRRRRSVIEDLLFTSVESVGLPTDDAYKNCTAGPL
Beaudette IBV p65               STGEFNISLLLTTPSSRRRRSVIEDLLFTSVESVGLPTDDAYKNCTAGPL
Beaudette IBV CK                STGEFNISLLLTNPSSRRKRSLIEDLLFTSVESVGLPTNDAYKNCTAGPL
Beaudette IBV                   STGEFNISLLLTNPSSRRKRSLIEDLLFTSVESVGLPTNDAYKNCTAGPL
Beaudette IBV-EP3               STGEFNISLLLTTPSSRRRRSFIEDLLFTSVESVGLPTDDAYKNCTAGPL
IBV M41                         STGEFNISLLLTTPSSPRRRSFIEDLLFTSVESVGLPTDDAYKNCTAGPL
IBV Cal99                       STGDFNISLLLTPPSSTTGRSFIEDLLFTSVESVGLPTDDAYKKCTAGPL
IBV Partridge/GD/S14/2003       STGDFNISLLLTPPSSPSGRSFIEDLLFTSVETVGLPTDAEYKKCTAGPL
IBV SAIBK                       STGDFNISLLLTPPSSPRGRSFIEDLLSTSVETVGLPTDAEYKKCTAGPL
IBV BJ                          TTGEFNISLVLTQPSSPSGRSFVEDLLFTSVETVGLPTDAEYKKCTAGPL
IBV LX4                         STGEFNISLLLTPPSSPSGRSFVEDLLFTSVETVGLPTDAEYKKCTAGPL
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C

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SARS Tor2                       GG--FNFSQILPDPLKPTKRSFIEDLLFNKVTLADAGFMKQYGECLGDIN
SARS Urbani                     GG--FNFSQILPDPLKPTKRSFIEDLLFNKVTLADAGFMKQYGECLGDIN
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B

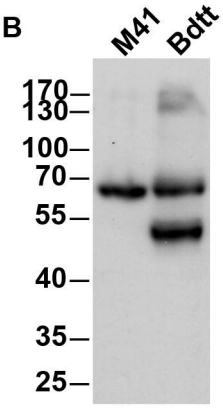

Fig. S2. Identification of additional cleavage sites in the coronavirus spike protein. (*A*) Multiple-sequence alignment of IBV S sequences in the vicinity of the proposed S2′ position. Sequence alignment was performed with Clustal W2. Basic residues in the S2′ position are shown in bold. (*B*) Western blot analysis of IBV M41 and Bdtt using an anti-S2 monoclonal antibody to detect the C-terminal part of the spike protein. (*C*) Multiple-sequence alignment of selected SARS-CoV S sequences in the vicinity of the proposed S2′ position. Sequence alignment was performed with Clustal W2. Basic residues in the S2′ position are shown in bold.

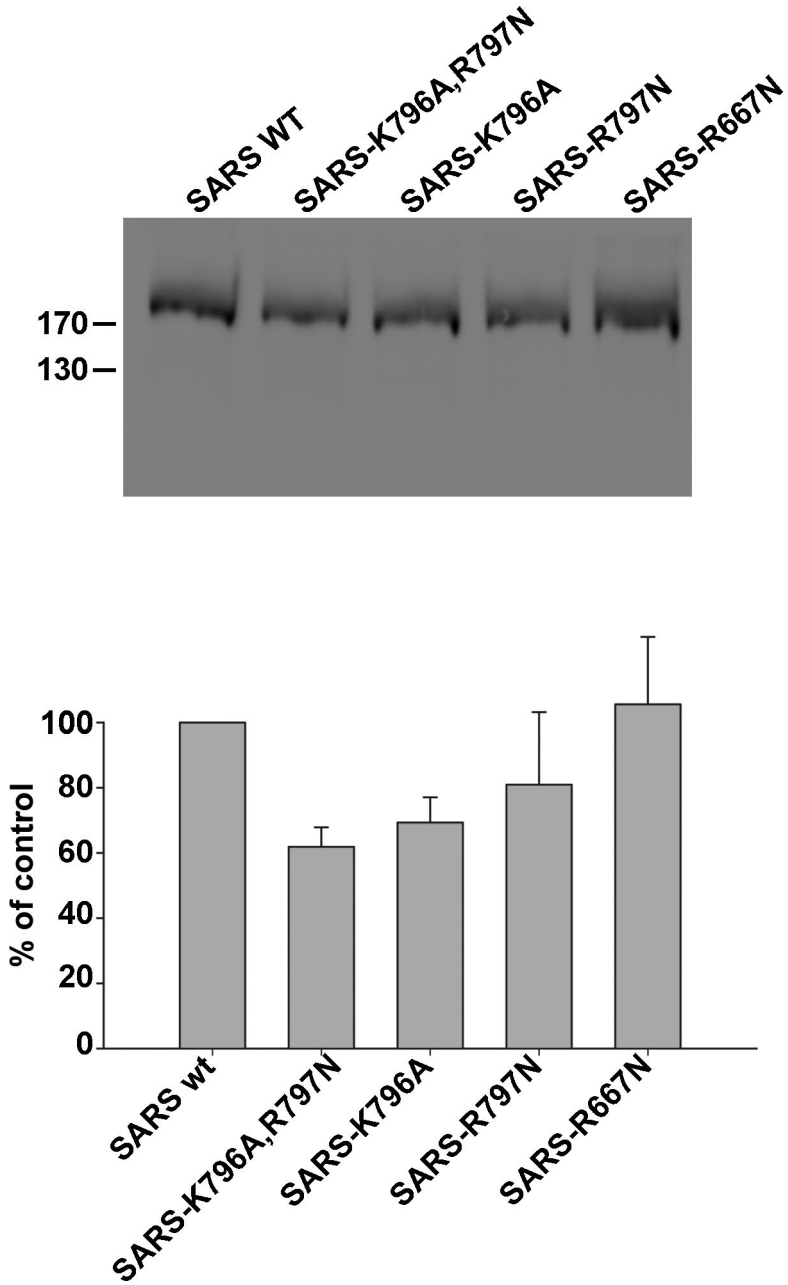

Fig. S3. Cell surface expression of SARS-CoV S2′ mutants. (*Upper*) BHK cells expressing expressing SARS-CoV S wild type (SARS wt) or S2′ mutants were analyzed for cell surface expression following surface biotinylation at 4 °C. Biotinylated proteins were precipitated by streptavin-agarose beads and analyzed by Western blot analysis. The different SARS spike proteins were detected with an anti-C9 antibody. (*Lower*) Quantification of cell surface expression. Results are presented as the percentage of expression of the wild-type protein at the cell surface.

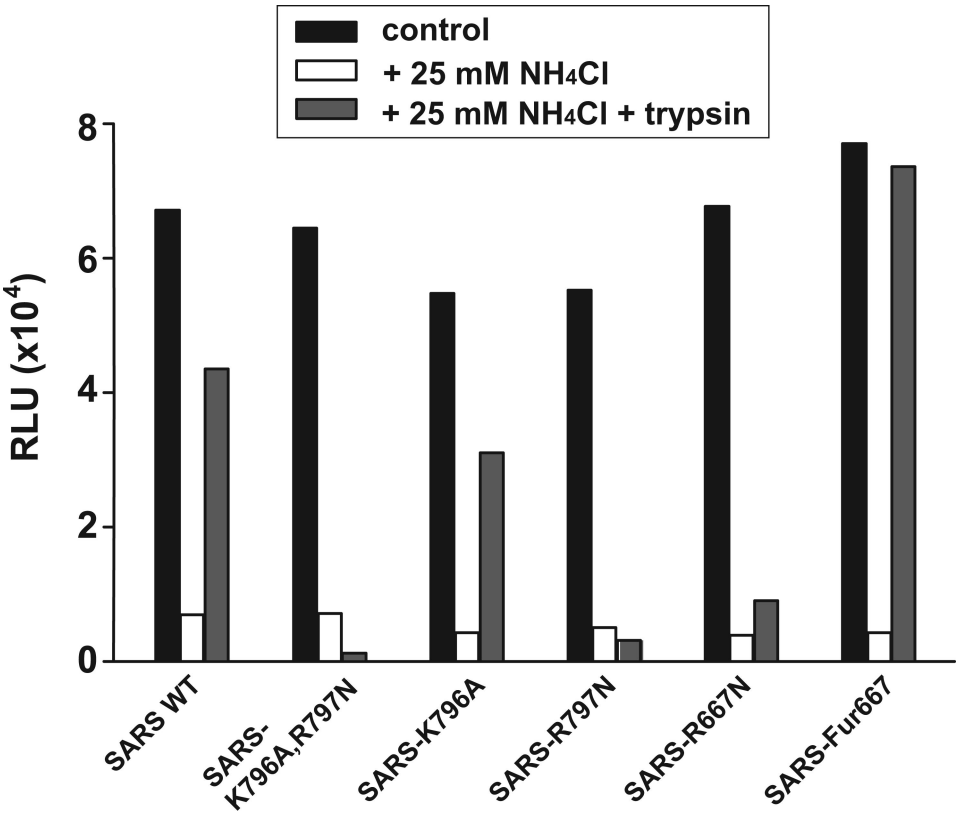

Fig. S4. Pseudovirion assays of SARS-CoV entry via trypsin-primed fusion in the presence of endosomal inhibitors. BHK cells coexpressing ACE2 and DC-SIGN were pretreated for 1 h with 25 mM NH_4Cl or remained untreated. Virions pseudotyped with the SARS-CoV S wild type (SARS wt) or the different mutants were bound at 4 °C for 2 h in presence of drug. Viral entry was induced by a 5-min treatment of the cells at 37 °C with either 3 μg/mL or 5 μg/mL trypsin. The results are expressed as relative light units (RLU).

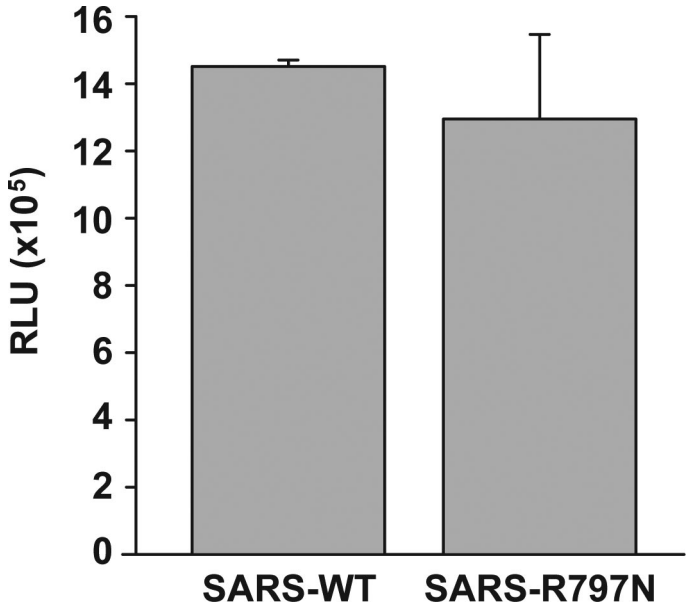

Fig. S5. R797 is not a critical residue for SARS-CoV entry via the endosomal pathway. BHK cells coexpressing ACE2 and DC-SIGN were transduced with the SARS-CoV S R797 mutant. The results are expressed as relative light units (RLU). Error bars represent the standard error of the mean for 3 independent experiments.